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Abstract. We hypothesized that inflammation results in detectable alteration of body odor and that traumatic brain injury (TBI) might similarly produce volatile metabolites specific to injury. Using an animal model, we first trained biosensor mice to distinguish between urine odors from lipopolysaccharide-treated and control mice. Lipopolysaccharide (LPS) is a general elicitor of inflammation. Trained biosensors could distinguish between the odors of LPS-treated and control mouse urine. Chemical analyses further demonstrated that LPS-induced inflammation results in alteration of urine volatiles. Importantly, urine samples collected many days following LPS-administration were discriminable. Thus, odor differences were not produced by acute effects of LPS-treatment (e.g. dehydration); nor were they likely related to changes in cytokines which typically occur within hours of LPS exposure and return to normal within 24 hours. We similarly demonstrated odor alteration due to treatment with LPS in humans. Urine samples collected from humans receiving a small dose of LPS (or control) were subjected to discrimination tasks by a human sensory panel as well as chemical analyses. Both assays suggested that treatment with LPS results in a detectable alteration of urine volatiles.

Odor changes resulting from TBI were also evaluated using an animal model. Because both LPS and TBI elicit inflammatory processes and LPS-induced inflammation induces body odor changes, we hypothesized that (1) TBI would induce a distinct change in body odor and (2) this change would resemble the change induced by LPS. Mice receiving surgery and lateral fluid percussion injury (LFPI) or surgery without brain injury were employed as urine donors. Biosensors trained to discriminate LPS-treated mouse odors from control odors did not generalize this learned response to LFPI (TBI) urine. However, a different panel was successfully trained to discriminate between urine odors of LFPI treated mice compared to control (surgery only) mouse urine odors. These results demonstrate that TBI does result in detectable alteration of body odor and further indicates, contrary to our initial hypotheses, that these changes differ from LPS-induced inflammation. These findings were confirmed by chemical analyses of TBI and sham urines which demonstrated that volatiles related to TBI-induced odor change differed from the volatile compounds that were altered by LPS administration. From these experiments, we conclude that both TBI and LPS-induced inflammation alters urine volatiles and that these alterations are specific to the two treatments.

Table of Contents

1. INTRODUCTION:

Chemical signals are the primary form of social communication for many species (Brennan & Kendrick 2006, Johnston 2003, Kelliher 2007). Although most research has been devoted to communication of social messages such as sex, age, and individual identity, volatile odorants may also communicate information about an animal's health status (Kavaliers *et al.* 2005, Moser & McCulloch 2010, Penn & Potts 1998). The mechanisms underlying changes in body odor caused by disease are poorly understood and the specificity of odor changes to a specific disease has rarely been explored. Immune function represents an interesting pathway for diseases to alter body odor (Beauchamp & Yamazaki 2005, Beauchamp *et al.* 1985, Brown & Eklund 1994). Based on this reasoning, we propose that chemical signals, acting through small volatile molecules (that is odorants), can be used to monitor immune activation and inflammation in humans and other animals. The ultimate goal of this work is to develop biosensors and chemometric approaches that can be successfully used to 'eavesdrop' on metabolic processes associated with inflammatory processes – such as traumatic brain injury.

2. KEYWORDS:

Biosensor; Body Odor; Human; Inflammation; Lipopolysaccharide (LPS); Mouse Model; Traumatic Brain Injury (TBI); Volatiles

3. OVERALL PROJECT SUMMARY:

Approval to amend the milestone deadlines was received on 05-Apr-13. The following schedule reflects approved changes:

Milestone 1 (Experiment 2) – Y1Q3, 15-Jul-13
Milestone 4 (Experiments 5, 6) – Y1Q4, 15-Oct-13
Milestone 2 (Experiment 1) – Y2Q1, 15-Jan-14
Milestone 3 (Experiments 3, 4) – Y2Q2, 15-Apr-14

A no-cost extension was granted 10-Mar-2014 to allow completion of Milestone 3. All milestones were completed by 14-Sep-14.

Milestone 1. Demonstration that lipopolysaccharide (LPS) induces odor changes in the mouse model using both animal biosensors and chemometric analyses was completed in Y1Q4. These results demonstrated that urine collected following treatment with LPS can be discriminated from urine collected from control subjects (treated with phosphate-buffered saline, PBS) on the basis of odor. Importantly, training was performed using urine samples collected 11 to 14 days post-treatment. Thus, the source of these odors was not related to acute symptoms of inflammation (such as dehydration) which lapse earlier than the urine collection date.

In rewarded training trials, mouse biosensors achieved 93% accuracy in their discrimination of LPS from PBS urine samples (Experiment 2). Following training, unrewarded generalization trials were conducted with LPS and PBS urine collected from

novel donors. Accuracy significantly greater than 50% indicates that a biosensor is discriminating LPS from PBS, not simply discriminating between individual donor odortypes used during training. Each of the six biosensors performed significantly better than chance in the LPS versus PBS generalization trials (Figure 1). Cumulatively, biosensors responded with 75% accuracy.

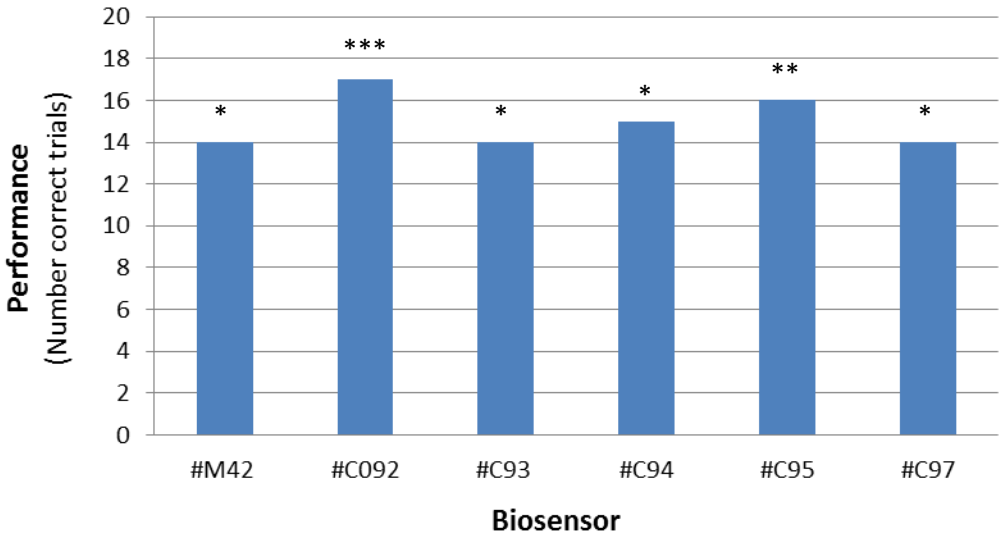

Figure 1. Mouse biosensor performance in 20 generalization trials for each trained mouse. Trials were divided by urine collection day (Days 11-14) into 4 sessions, each of which included 5 generalization trials with urine from a single collection day (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

Chromatographic data from headspace gas chromatographic analyses of mouse urines also indicated that treatment with LPS induced changes in volatile profiles in the mouse model, consistent with the mouse biosensor results reported above. Urine samples were collected from mice treated with either a single LPS or single control (PBS) injection and the chromatographic data from a total 514 urine samples (including multiple collections from each donor across multiple collection days) were subjected to linear discriminant analysis.

Using only three peaks (each peak being a chromatographic response from an individual volatile chemical), 18 of 20 samples collected from mice receiving LPS and 18 of 20 samples collected from mice receiving control were correctly categorized. The error estimate upon cross-validation was 10.0%.

Milestone 4. Characterization of changes in human body odor in response to treatment with LPS by human sensory panel and by chemometric analyses was completed in Y1Q4. The results of Experiment 5 ("Psychometric analyses of odor samples from LPS-treated human donors") suggested that LPS injection induces changes in the volatiles emitted by human urine odor, which are detectable to humans and differ from the

changes that occur naturally over the course of a day. Results of Experiment 6 (“Chemometric analyses of odor samples from LPS-treated human donors”) strongly supported these suggestive sensory results.

Human biosensor evaluation of human urine suggests that lipopolysaccharide (LPS) injection induced changes in the urine volatiles which differ from naturally occurring changes in odor over the course of a day. Twenty-one human biosensors each performed two within-donor, three-alternative, forced-choice (3AFC) discrimination tasks. One task included urine samples from LPS-treated urine donors and the other included samples from urine donors treated with a control solution (phosphate-buffered saline). Each discrimination task was comprised of 11 trials, and each trial contained three urine samples from the same donor: two identical 'Lure' samples and one 'Target' sample. Humans were instructed to choose which one of the three samples differed from the other two. Thus, when attempting to identify the post-injection LPS urine from among the two identical pre-treatment urines (or the post-injection control sample from among two identical pre-treatment urines), the rate of correct identification by chance was 33%. The order of LPS and Control discrimination tasks was counter-balanced across human biosensors.

Human biosensors discriminated post- from pre-injection urine samples of donors injected with LPS significantly more often than could be expected by chance ($x=4.45$, $n=20$, $p<0.05$) (*Fig. 3*). Human biosensors also discriminated post- from pre-injection urine samples of control donors ($x=5.75$, $n=20$, $p<0.001$; Figure 2). However, discrimination performances with LPS and PBS post-injection Target samples were not correlated ($n=20$, $r=-0.069$), as tested with the Pearson's correlation.

These results are consistent with the hypothesis that LPS injection alters the changes in human urine odors normally seen over the course of the day. Chemical studies provide stronger support for this hypothesis, as described below.

Chromatographic data from headspace GC/MS analyses (Experiment 6) of the same human urine were subjected to linear discriminant analysis. Using three chromatographic peak responses, 15 of 19 samples collected from LPS-injected Donors and 11 of 13 samples collected from PBS-injected Donors were correctly categorized. The cross-validation error rate was 20.9%.

Together, these results suggest that LPS injection alters the changes in human urine volatiles normally seen over the course of the day in a predictable manner. Differences in human urine odor before and after lipopolysaccharide (LPS) injection are recognizable to human subjects, and can be characterized by changes to as few as three chemical components. The chemical components identified in the mouse model studies and the human studies were not the same.

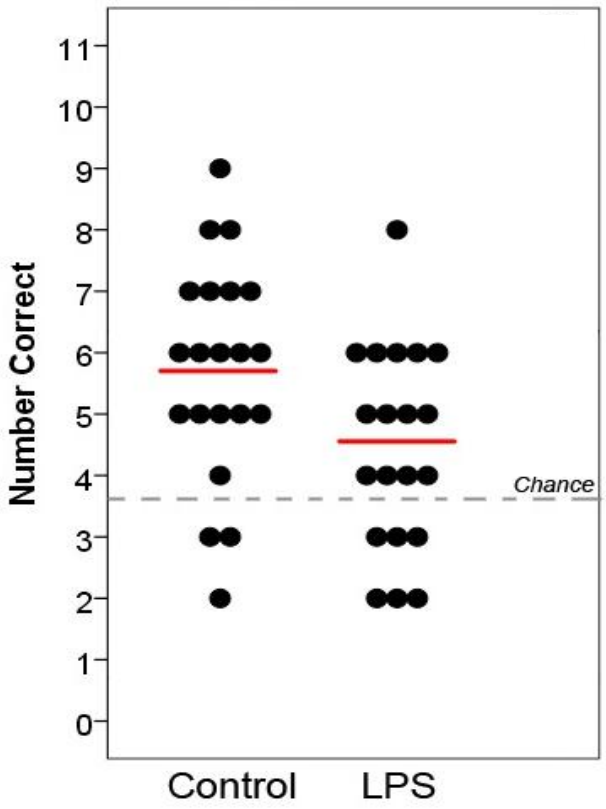

Figure 2. Human subject performance in LPS and control discrimination tasks, as measured by number of correct trials. All subjects (n=20) performed both tasks, and group means are indicated by red lines. Discrimination performances in both the LPS (x=4.45 correct trials) and control (x=5.75) conditions were significantly better than chance performance ($p<0.05$).

Milestone 2. Investigation of the time course of LPS-induced odor change (Experiment 1) in the mouse model was completed in Y2Q1. Chemometric evaluation was completed in Y1Q4 and mouse biosensor evaluation was completed in Y2Q1.

Chromatographic data from 514 urine samples were subjected to principal components analysis. Principle components were modeled versus day of urine collection (post-treatment). When fitted to logarithmic functions, results demonstrate that urine volatile differences between the LPS and control treatment persist for approximately 35 days (Figure 3).

A panel of mice was trained to discriminate LPS and control urines collected 11 – 14 days after treatment. In generalization trials, these mice discriminated urines collected from 5 to 28 days post treatment (Figure 4). Although LPS-related odor was persistent for four weeks, it was no longer discernable in urine collected five months post LPS exposure (no samples were evaluated between these two time periods). Rapid onset of odor change (within days of exposure) suggests that innate immunity processes were responsible for the volatile metabolites detected by mice. However, we do not know the underlying

physiology responsible for the persistence of the LPS-induced change in urine odor volatile profile.

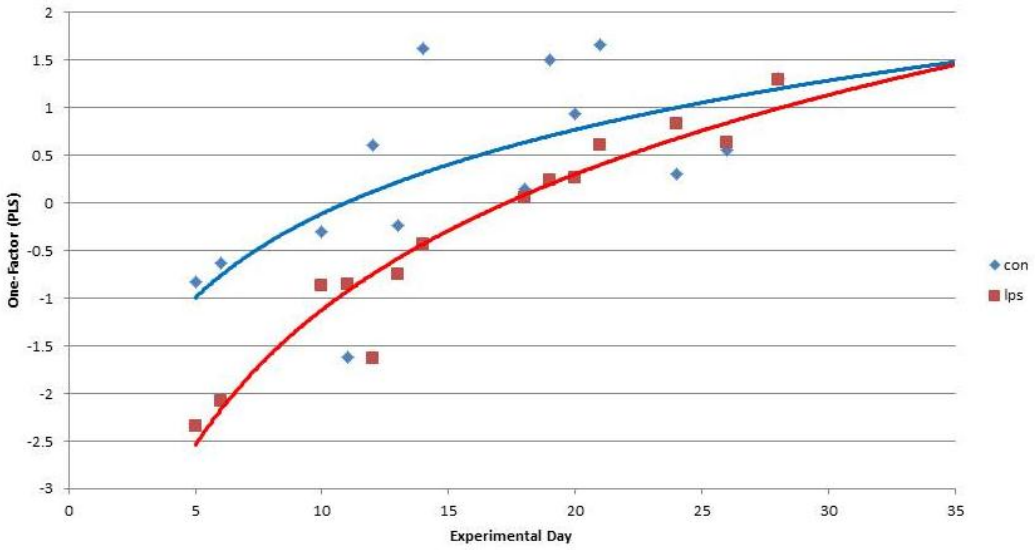

Figure 3. Mouse urine volatile differences between the LPS and control treatment persist for approximately 35 days.

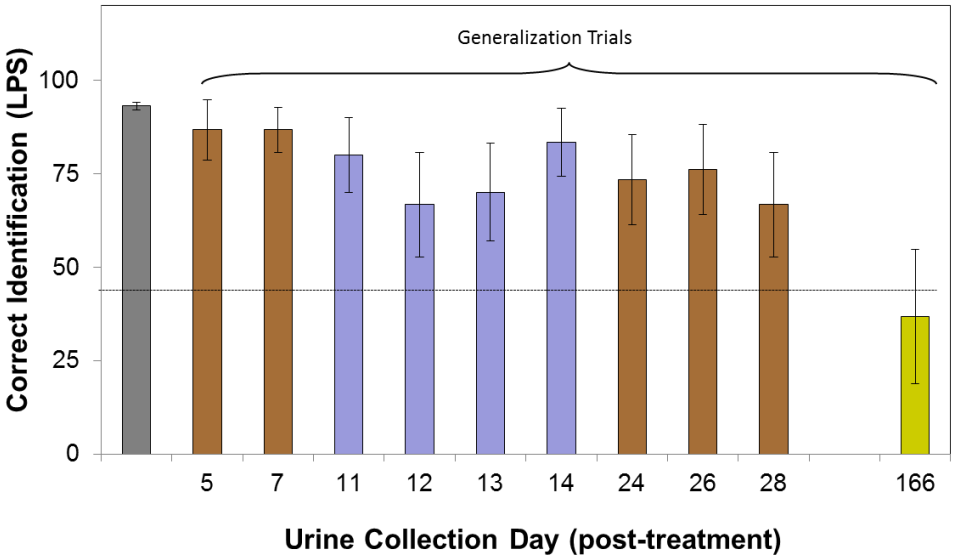

Figure 4. Trained biosensor responses to pair-wise offering of LPS and control urines. Urines collected days 11 – 14 were used in rewarded training trials (gray bar). Colored bars represent unrewarded generalization trials.

Milestone 3. Investigation of altered body odors in the mouse model of traumatic brain injury (TBI) using both animal biosensors (Experiment 3) and chemometric analyses (Experiment 4) was completed by 14-Sep-14.

Biosensors trained to discriminate LPS and control urines (Experiment 1, above) were offered urine samples collected from LFPI (n = 7) and sham-treated (n = 5) mice using the Y-maze. In 60 trials, urine from LFPI urine was selected 34 times (57%; p = 0.366). This lack of discrimination by LPS-trained biosensors suggests that the chemicals contributing to the odor of LPS inflammation did not vary according to treatment in urines from LFPI (brain injury) and sham donors.

Multiple urine samples were collected from mouse donors 1 to 10 days following sham surgery (n = 19) or surgery and LFPI-induced brain injury (TBI; n = 20). A panel of five biosensors was trained to discriminate TBI and sham urines in the y-maze. All five biosensor mice demonstrated better than 80% concordance by selecting urines collected from TBI donors on the basis of odor. Generalization trials were conducted with urine from donors (different from donors used during training) collected 1 to 10 or 11 to 15 days following surgery. Results demonstrated that urine collected from mice 1 to 15 days following LFPI (TBI) can be discriminated on the basis of odor (Figure 5). Use of surgical controls for comparison in the Y-maze demonstrates that the source of these odors was not related to surgical pharmacology (e.g. anesthetics, analgesics) or surgical site inflammation.

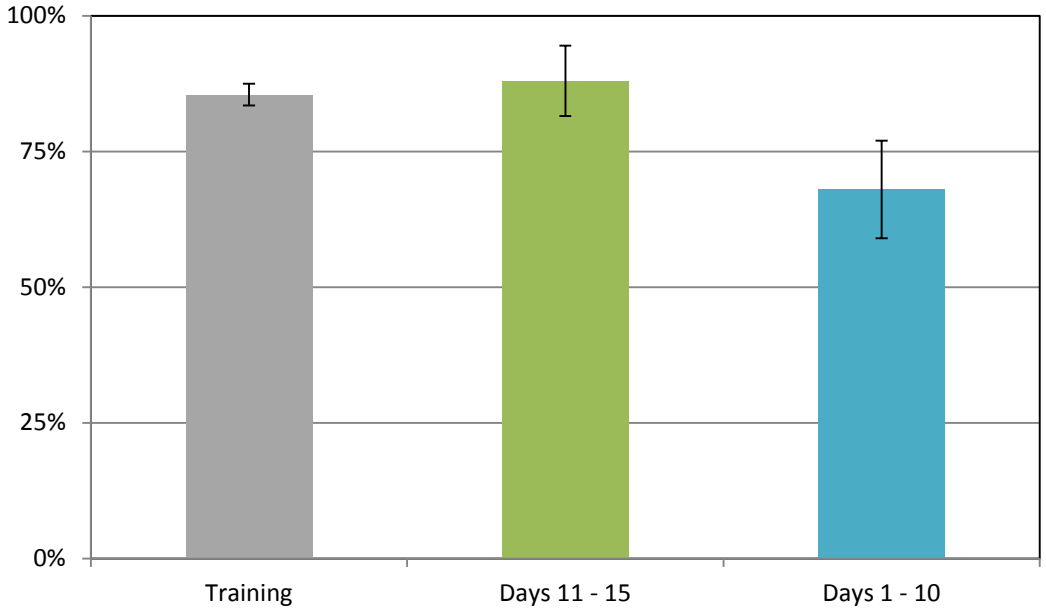

Figure 5. Mean responses of trained biosensors (with 95% confidence intervals) indicate LPI odor recognition during training and two generalization periods.

Chemical analyses of urinary volatiles indicated that mice could be accurately classified as receiving LFPI or sham surgery using a linear discriminant analysis (LDA) model employing chromatographic peak responses of four volatile chemicals. Fourteen of 15 (93%) LFPI and 12 of 14 (86%) sham donors were correctly classified. Peak responses from analyses of urines collected from three additional (not used in LDA model building) LFPI and three additional sham donors were used to predict treatment status using the LDA model. Three (of three) LFPI and two (of three) sham control were correctly classified from the peak responses of the four compounds. Chemical analyses further demonstrated that only one volatile compound was shared between the LPS classification model (employing responses of three compounds) and the LFPI classification model (employing responses of four compounds), further indicating that odor changes caused by LFPI differed from LPS-induced inflammation.

4. KEY RESEARCH ACCOMPLISHMENTS:

- Lipopolysaccharide (LPS) induces detectable changes in body odor as evidenced in mouse urine.
- LPS-induced changes to mouse urine odor can be explained by a model using only three peaks (chromatographic responses from three individual volatile chemicals).
- LPS induces predictable changes in human urine odor that can be detected by other humans.
- LPS-induced changes to human urine odor can be explained by a model using only three peaks (chromatographic responses from three individual volatile chemicals).
- Traumatic brain injury (TBI) induces detectable changes in body odor as evidenced in mouse urine. However, mice trained to discriminate LPS-treated mouse urine from control urine did not discriminate TBI urine from control urine indicating that the odors of the two treatments (LPS and TBI) differed.
- TBI-induced changes in mouse urine odor can be explained by a model using four chromatographic peak responses; only one of which is shared with the discriminant model for LPS-induced inflammation in mice.

5. CONCLUSION:

We conclude that both TBI and LPS-induced inflammation alters urine volatiles. Furthermore, alterations of volatile metabolites produced by administration of LPS differ from those caused by LFPI-induced brain injury (TBI). Taken together, these results suggest that occurrence of TBI (and recovery from injury) can potentially be monitored via evaluation of volatile metabolites with some specificity.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

a. Publications

(1) Lay Press: Nothing to report.
(2) Peer-Reviewed Scientific Journals: Nothing to report.
(3) Invited Articles: Nothing to report.

(4) Abstracts:

Kimball, B.A. and Beauchamp, G.K. Eavesdropping on Immunity. Association of Chemoreception Sciences 46th Annual Meeting. Bonita Springs, FL 04/14.

b. Presentations (* indicates associated manuscript)

Gary K. Beauchamp, "Odor Signatures of Inflammation." Annual Meeting of Monell Sponsors, Philadelphia, PA. 08 October 2013.

Bruce A. Kimball, "Eavesdropping on Immunity." Contributed poster presentation; Association of Chemoreception Sciences 46th Annual Meeting. Bonita Springs, FL 04/14.

Bruce A. Kimball, "Volatile Markers of Brain Injury." Annual Meeting of Monell Sponsors, Philadelphia, PA. 01 October 2014.

7. INVENTIONS, PATENTS AND LICENSES:

The inventors have submitted an invention disclosure entitled "Urinary biomarker for mild traumatic brain injury." The disclosure is currently being reviewed by the technology transfer offices at Monell, Children's Hospital of Philadelphia, and USDA.

8. REPORTABLE OUTCOMES:

The disclosure referenced above contemplates intellectual property protection of a series of biomarkers that could be used to diagnose mild traumatic brain injury. A manuscript intended for publication in a peer-reviewed journal is currently in preparation.

9. OTHER ACHIEVEMENTS: Nothing to report.

10. REFERENCES:

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11. APPENDICES: None.